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Whole mount *in situ*hybridization on freshwater planaria

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▼Freshwater planaria (Plathyhelminthes, Turbellaria, Tricladida) are famous for their phenomenal ability to regenerate: the morphological pattern of the whole organism can be reconstituted from anypiece of tissue (Ref. 1). Planarian regeneration has been rather well described at anatomical, histological and cytological levels but biochemical and molecular studies of this process are only just beginning (Ref. 2).

Subtractive hybridization made it possible to identify several nucleotide sequences differentially expressed in the course of planarian regeneration (Bogdanova *et al.*, manuscript in preparation). One of them, named *scarf*, was found to be much more abundant in the tail blastema than in the head. Further studies have shown the uneven distribution of transcripts of these tissue-specific nucleotide sequences along the anteroposterior axis of an intact planarian body. In addition, a number of planarian homeobox-containing genes, related to those involved in the body pattern formation in other animals, have also been described (Ref. 3, 4, 5). Accumulation of such genetic markers leads to the necessity for a sensitive and accurate analysis of their expression in intact and regenerating planaria.

Whole mount *in situ* hybridization is now commonly used for gene expression analysis (see Ref. 6 for a detailed description of this method). Unfortunately, this technique has proved to be inadequate for planaria. It is thought that some of the chemicals involved (i.e., the labelled riboprobe itself) do not penetrate planarian tissue very well. The same problem affects *in situ*hybridization performed on many other tissues (e.g., lens, spleen, late-stage mice embryos).

To overcome this problem, we attempted to use the maceration technique, initially introduced by David for *Hydra*(Ref. 7). Usually, it is used to obtain a homogenous cell suspension for cytometrical purposes and statistical estimation of cell state (e.g., frequency of mitosis). Baguna *et al.* have successfully applied this technique to planaria (Ref. 8). According to their data, planaria incubated overnight in a solution containing acetic acid, methanol, glycerine and water are completely macerated. We assumed that the same incubation, but with less fixative added to prevent complete maceration, could increase the permeability of planarian tissue. We combined such a treatment with the standard *in situ* hybridization protocol for *scarf* expression analysis in intact specimens of the planarian *Dugesia tigrina*.

The results of *scarf* expression analysis by means of reverse transcription–polymerase chain reaction (RT–PCR) are shown (Fig. 1). The specimen was cut transversely into six fragments which were analyzed independently. A spatial distribution of *scarf* mRNA in the intact planarian was revealed: none was found in the head, the most occurred in the second part, with a rather steady decline towards the posterior.

We had experience of whole mount *in situ* hybridization under standard protocol on sagittally dissected planaria (Fig. 2a, b). The signal appeared only in tissues closely adjacent to the surface of the cut. Almost all staining was located in places where, according to RT–PCR data, the highest level of *scarf* expression was expected, but the lack of *scarf* expression in posterior parts of the body did not correspond to gradient revealed by RT–PCR (compare Fig. 1 and Fig. 2b). Without dissection, no signal at all was observed (Fig. 2a), perhaps due to the collagen-containing basal membrane which underlies the outer layer of cells – the epidermis.

The pattern of *scarf* expression in intact *D. tigrina*, obtained according to our whole mount *in situ* hybridization protocol, is shown (Fig. 2d, e). A 'horse-shoe' pattern is seen in the 'neck' region, extended posteriorly by two longitudinal lateral branches of discrete cell clusters intensely

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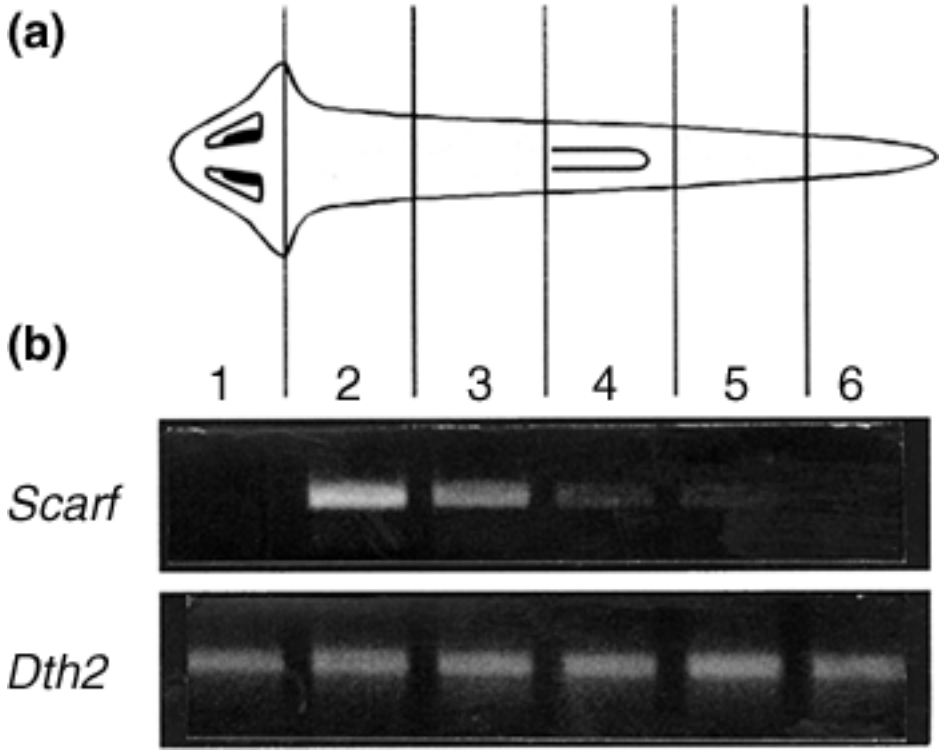

FIGURE 1. RT–PCR analysis of *scarf* expression in the planarian body. (a) Diagram showing the dissected body fragments taken for analysis. (b) RT–PCR analysis of *scarf* and *Dth-2*expression (*Dth-2* transcripts are known to be evenly distributed in parenchymal tissue along the anteroposterior axis of the planarian body; Ref. 11). Total RNA samples were reverse-transcribed according to the standard protocol and subjected to PCR with oligonucletide primers for *scarf* (5′-CTTGTTGGGTTAATTTTCCAACT-3′ and 5′-CATTTAATCACATTCGTCCTC-3′) and *Dth-2* (5′-TTGGTTCCAAATCGGCTTCC-3′ and 5′-CCAGAACGTGAGCATTTGGC-3′) Each PCR cycle included 95°C for 10 s, 58°C for 15 s and 72°C for 40 s. Nineteen cycles were performed in the case of *scarf*and twenty five cycles in the case of *Dth-2*.

expressing *scarf*. The density of these clusters decreases in the posterior direction. No expression of *scarf* was detected in the head region. These data correlate with the results of RT–PCR analysis (Figures 1 and 2). No signal was obtained in the negative control experiment, involving sense *scarf* RNA as a riboprobe (Fig. 2f).

We also undertook a histological analysis of a a stained specimen. This was paraffin embedded and cut transversely in 12 μm sections, which were mounted in Canada balsam without any additional staining. Such sections through different levels of the planarian body are shown (Fig. 3).

A great variety of maceration cocktails are in general use for histology (Ref. 9), including those for individual types of tissue (such as alcohol–hydrochloric acid for isolation of renal tubules, or water–picric acid–acetic acid for disassociation of striped muscle). We believe that the introduction of maceration elements into the standard fixation procedure might also give good results for other objects with low tissue permeability.

Whole mount *in situ* hybridization procedure

In all experiments we used *D. tigrina G.*(asexual race), starved for two weeks. The water in the Petri dish containing living planaria was replaced with Locke solution (0.8% NaCl, 0.02% KCl, 0.02% $CaCl_2$, 0.02% $NaHCO_3$) for 2 min to induce the release of mucus (Ref. 10), then with distilled water for 5 min, and finally with maceration mixture (distilled water, methanol, glycerine and glacial acetic acid, 14:3:2:1, respectively) for 30 s. Planaria were then transferred to 2 ml plastic Eppendorf tubes containing 1 ml of maceration mixture with 0.5, 1.5, 2 or 3% formaldehyde and were left rocking horizontally at 4°C overnight. After such incubation, planaria in 0.5% formaldehyde were macerated almost completely, while the higher concentrations of formaldehyde allowed specimens to keep their natural shape. The latter specimens were then fixed in 4% formaldehyde in phosphate-buffered saline (PBS) at 4°C for 2 h. Then the *in situ* hybridization procedure according to Harland (Ref. 6) was performed.

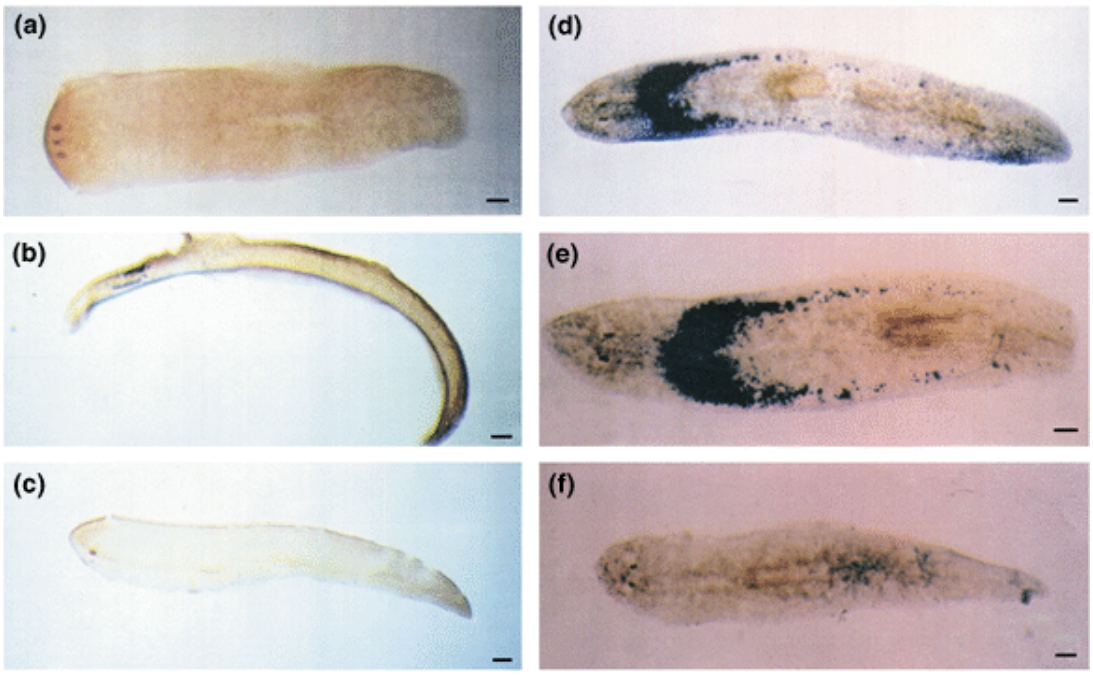

FIGURE 2. Analysis of*scarf* expression in the intact planarian by whole mount *insitu* hybridization. (a–c) *In situ* hybridization was performed following the standard protocol (Ref. 6). (a) *In situ* hybridization with *scarf* antisense riboprobe without dissection of the planarian body. Signal was not detected. (b, c) *In situ* hybridization of sagittally cut planarian with antisense (b) and sense (c) digoxigenin-labelled *scarf* riboprobe. (d–f) *Insitu* hybridization was performed as described in the text using an antisense (d, e) or sense (f) *scarf* riboprobe. The maceration mixture used contained 2% formaldehyde. All specimens are viewed from the dorsal side. Bar = 200 μm.

The *scarf* fragment (800 bp) was cloned into pCDNA2 vector (InVitrogen). RNA probes were synthesized using the standard protocol and purified by the RNeasy (Quiagen) system. To hydrolyze the probe into 300 bp fragments, 1 μg of digoxygenin-labelled RNA was incubated in 30 μl of 40 mM sodium bicarbonate–60 mM sodium carbonate, 40mM Na_2CO_3 (60°C for 45 min). Then 1 ml of hybridization buffer [5 × SSC, 50% deionized formamide (Sigma), 1 × Denhart solution, 0.3% CHAPS (Sigma), 0.1% Tween 20 (Sigma), 5mM EDTA and 1mg/ml Torula tRNA (Boehringer Mannheim)] was added directly to the hydrolyzing solution.

After dehydration–rehydration of the fixed planaria in PBS–methanol series, they were treated with proteinase K (Boehringer Mannheim) (20 μg/ml for 45 min at room temperature). Specimens were prehybridized in 300 μl of hybridization buffer for at least 4.5 h (up to overnight) at 58°C, then the buffer was replaced by a fresh portion containing the probe. Hybridization was carried out overnight at the same temperature. The unhybridized riboprobe was consequently washed off (2 × SSC, 0.3% CHAPS; 1 × SSC, 0.3% CHAPS and two changes of 0.2 × SSC, 0.3% CHAPS at 58°C, 30 min each wash); RNase steps were omitted. Specimens were then preblocked in 10% heat-inactivated sheep serum (Sigma) in PBT (PBS containing 0.1% Triton X-100 and 2mg/ml bovine serum albumen) for 1 h at room temperature. Anti-digoxygenin antibody (Boehringer Mannheim) (0.75 U/μl) was preabsorbed in 1% sheep serum solution in PBT in the presence of planarian powder (acetone-extracted homogenized tissue; 1 mg of powder per 1 μl of antibody) for 1 h at 4°C. Antibody incubation (1/2000 dilution in 1% sheep serum) was carried out overnight at 4°C. PBT washes, removing the excess of antibody, and NBT/BCIP staining were performed as described by Harland (Ref. 6). The staining was terminated after about 30 min from the start of a phosphatase reaction. Stained specimens were transferred into methanol and mounted in 2:1 bensyl bensoate:bensyl alcohol.

The greater the concentration of formaldehyde in the maceration mixture, the less intense was the hybridization signal. However, the integrity of specimens appeared satisfactory for further histological analysis only for the higher of tested formaldehyde concentrations (2% and 3%). Thus, we found the concentration of 2% formaldehyde in the maceration mixture to be optimal.

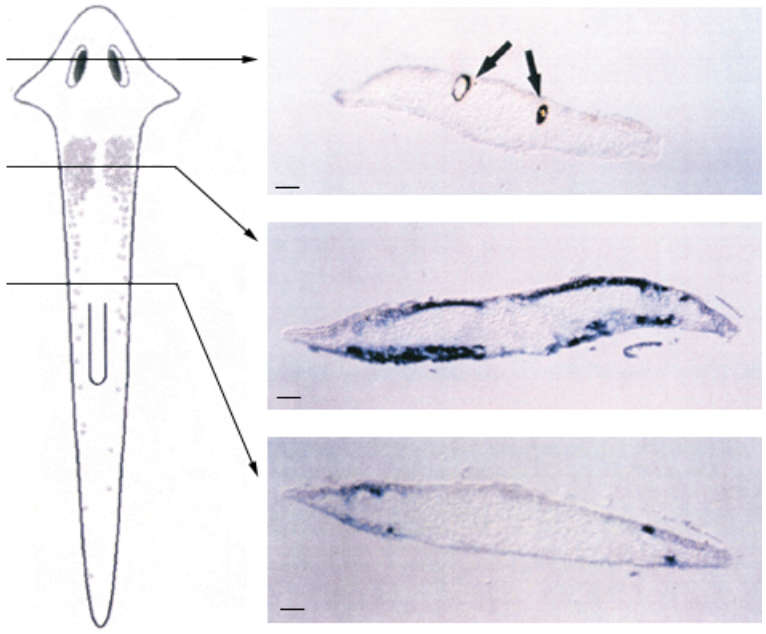

FIGURE 3. Histological analysisof the specimen after whole mount*in situ* hybridization with *scarf* probe. Transversal paraffin sections were made at the levels indicated on diagram on the left (*scarf* expression regions are shaded). All sections are shown dorsal side up. The signal is absent in the head (the eyes are indicated by arrows). In the 'neck' region, domains of *scarf* expression are observed in dorsal and ventral layers of parenchymal tissue, with none in the inner cell population corresponding to the gut (compare with the 'half-mount' specimen in Fig. 2b). On the posterior-most section, the signal is restricted to lateral parenchymal cells that correspond to the longitudinal 'branches' of labelled cell clusters (see Fig. 2 d,e). Bar = 50 μm.

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Products Used

pCDNA2 vector: pCDNA2 vector from Invitrogen
cDNA: cDNA from Clontech Inc
RNeasy: RNeasy from QIAGEN GmbH
formamide: formamide from Sigma
Formamide: Formamide from Life Technologies (Gibco BRL)
CHAPS: CHAPS from Sigma
Tween 20: Tween 20 from Sigma
Torula tRNA: Torula tRNA from Boehringer Mannheim
proteinase K: proteinase K from Sigma
proteinase K: proteinase K from Boehringer Mannheim
proteinase K: proteinase K from QIAGEN GmbH
Proteinase K: Proteinase K from PE Applied Biosystems
sheep serum: sheep serum from Sigma
Anti-digoxigenin antibody: Anti-digoxigenin antibody from Boehringer Mannheim